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Measurement of Protein Biomass by Fourier Transform Infrared–Photoacoustic Spectroscopy

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A relatively new analytical technique, Fourier transform infrared–photoacoustic spectroscopy (FTIR–PAS), provides spectra of bacteria, fungi, and other microorganisms in solid states not suitable for conventional absorption spectroscopy. In this paper the feasibility of quantitative measurement of protein biomass on solid substrates by FTIR–PAS is examined and discussed. By measuring photoacoustic absorption bands from amide groups in the protein of microorganisms, the increase in biomass that occurs during growth was monitored directly and accurately. Incorporation of polyacrylonitrile into the sample as an internal standard was shown to be a convenient method for improving both the reliability and the range of detection by photoacoustic spectroscopy. Results of FTIR–PAS measurements of known quantities of microbial mass in simulated growth experiments suggest that the technique may be especially suitable for assays of microorganisms used in solid-state biosyntheses of drugs, hormones, and other biological agents.

Despite the variety of assays currently used to measure biomass formed during fermentation on substrates such as cellulose (1), quantitative analysis of microbial growth on solid surfaces is still extremely problematic. Present methods are cumbersome and may be accompanied by significant loss of the biomass being measured. Such analyses may be classified as either degradative (2, 3) or disruptive (4, 5), and consequently they are often not highly reproducible.

In recent years a relatively new analytical technique, photoacoustic spectroscopy (PAS),[2] has been developed as a tool for investigations of solids and other materials that are difficult to study by conventional optical analyses (6–8). The infrared photoacoustic effect with solids is simple in principle. A sample is placed in a closed cell containing a sensitive microphone, and a beam of infrared radiation is admitted through a window in the cell to irradiate the sample. When the solid absorbs infrared radiation, it becomes heated and warms the layer of air or gas surrounding the sample. The warmed gas expands and produces increased gas pressure inside the acoustically sealed cell. When the infrared beam is blocked off, the sample cools and the gas pres-

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

[2] Abbreviations used: PAS, photoacoustic spectroscopy; FTIR, Fourier transform infrared; BSA, bovine serum albumin; PAN, polyacrylonitrile.

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sure in the cell returns to its original level. If the infrared beam is periodically chopped or modulated at a frequency within the response range of the microphone, the periodic heating and cooling produce gas pressure waves in the cell that can be detected as sound. The pitch of the sound produced is the same as the modulation frequency, but the volume of the sound depends upon the amount of radiation absorbed by the sample.

Thus when a sample is exposed to a modulated infrared beam, radiation is absorbed at various frequencies in different amounts, and the resulting acoustic signal detected by the microphone has the same general form as the original infrared absorption signal. In Fourier transform infrared photoacoustic spectroscopy (FTIR–PAS) the form of this signal is an interferogram, which is subsequently transformed mathematically into a photoacoustic absorption spectrum that is representative of the infrared absorption spectrum of the sample. In contrast to optical spectroscopy, only absorbed radiation produces photoacoustic signals. Hence, any radiation that is lost due to scattering is of no consequence, and samples need not be transparent. More rigorous treatments of photoacoustic theory are presented elsewhere (9–11).

FTIR–PAS has been proven a useful tool for qualitative analyses of solids (12, 13). It is especially suitable for granular or filamentous materials which have large surface areas that produce high photoacoustic signal-to-noise ratios. However, even with the general theory of photoacoustics well established, there has been little application of the technique to practical quantitative analyses of solids. In an earlier paper the potential of FTIR–PAS for quantitative assay of fungal growth on cellulose was demonstrated and observed to possess several advantages in this type of assay (14): notably, the ease of sample preparation, preservation of biomass, and immunity to the light scattering that compromises quantitative analysis by transmission spectroscopy. Nevertheless, real uncertainties exist, and FTIR–PAS for quantitative analysis of solids, and of biomass in particular, is yet in its infancy. The purpose of the current investigations is to obtain basic information to advance this new technique toward the goal of practical application to biomaterial assays.

MATERIALS AND METHODS

Proteins. BSA (bovine serum albumin), alkaline phosphatase (calf intestinal mucosa), and lysozyme (chicken egg white) were purchased from Sigma Chemical Co. Cytochrome *c* was obtained from United States Biochemical Corp.

Microorganisms. The bacterium *Escherichia coli* strain HB101 was obtained from BRL (Gaithersburg, MD.) and grown in LB medium. The yeast *Saccharomyces cerevisiae* strain NRRL Y-2034 was obtained from the ARS Culture Collection (NRRL, Peoria, IL) and grown in YM medium. The eukaryotic, Chlorella-like green alga strain NC64A was a gift from Dr. James L. VanEtten. The filamentous fungus *Trichoderma reesei* strain QM 9414 was a gift from Dr. Mark A. Jackson.

Reagents. Polyacrylonitrile (PAN), Type A, was purchased from E. I. Dupont Co. Glutaraldehyde was obtained from Sigma Chemical Co. Ethanol and hydrochloric acid were of analytical reagent grade.

Filters. Durapore (polyvinylidene difluoride) GVWP 02500 filters were obtained from Millipore Co.

Preparation of PAN for internal standard use. PAN was washed with dilute HCl and dried. The insoluble polymer was suspended in distilled water by pulverizing the

powder in a WIG-L-BUG amalgamator with just enough water to form a homogeneous paste. The paste was then diluted with distilled water to 1.0% PAN concentration. Typical PAN suspensions remained stable for several days at room temperature.

Preparation of biomass samples for FTIR–PAS analyses. Suspensions of the proteins were prepared by pulverizing the powders and dispersing them in absolute ethanol. Finely divided protein particles remained suspended in ethanol for several minutes before sedimentation occurred. Suspensions of the microorganisms were prepared by gentle shaking in distilled water without pulverizing or disrupting the cells. These suspensions also remained stable for several minutes. All of the suspensions were reshaken just prior to use. Final protein concentrations in both the protein and the microorganism suspensions were determined by the method of Lowry *et al.* (4) following dilution to approximately 3.3 $\mu g/\mu l$.

To test the accuracy and reproducibility of FTIR–PAS measurement of biomass on a solid surface, a range of known weights of each protein and each microorganism was deposited with a known weight of PAN onto Durapore filters. This provided a series of simulated growth samples with increasing weights of protein containing a constant weight of the PAN internal standard for each biomaterial investigated. Reference samples were prepared by deposition of PAN onto filters in the same manner but without protein or microorganisms.

In a typical example, a 100-μl aliquot of an *E. coli* suspension containing 3.4 $\mu g/\mu l$ protein was pipetted into 10 ml distilled water on a Durapore filter in a sintered glass funnel. This was followed immediately by a 33.3-μl aliquot of the 1.0% PAN suspension pipetted into the water. The resulting dilute suspension of *E. coli* and PAN was stirred to mix well and then filtered by gentle suction. Thus a uniform layer of *E. coli* cells (340 μg protein) and PAN (333 μg) was deposited onto the surface of the filter. In a typical protein sample such as BSA the only difference from the *E. coli* example was the use of 10 ml absolute ethanol instead of distilled water to prevent loss of soluble protein through the filter. All samples were dried on the filters by vacuum desiccation at 50°C prior to testing.

Test samples for FTIR–PAS analyses were cut from the filters with a common paper punch and placed in 0.3-cm-deep sample cups in the PAS cell. The surface area of a punched-out sample was 1/8 the area of the total biomass layer deposited on the filter. Hence 1/8 of the deposited biomass was tested in each of up to four replicate test samples.

FTIR–PAS. Photoacoustic spectra were obtained with an Analect FX-440 photoacoustic cell in the sample compartment of an Analect FX-6260 spectrometer equipped with a TGS detector. Spectra were measured at 4-cm^{-1} resolution over the infrared range 400 to 4000 cm^{-1}. Interferograms were processed with trapezoidal apodization by using a Map-66 multiple-array data processor. The optical head, sample compartment, and PAS cell were purged with dry nitrogen to eliminate water-vapor and CO_2 interference. Spectra were generated from 300–500 interferometer scans that were signal averaged over 15–25 min accumulation time. All recorded FTIR–PAS spectra were smoothed at 21-point intervals but are otherwise presented uncorrected.

Scanning electron microscopy. Samples were fixed with 2% glutaraldehyde in 0.1 M Na_2HPO_4 (pH 7.2) for 30 min at room temperature. They were then dehydrated by sequential additions of ethanol, critical point dried, and examined with an ISI Model 55130 scanning electron microscope.

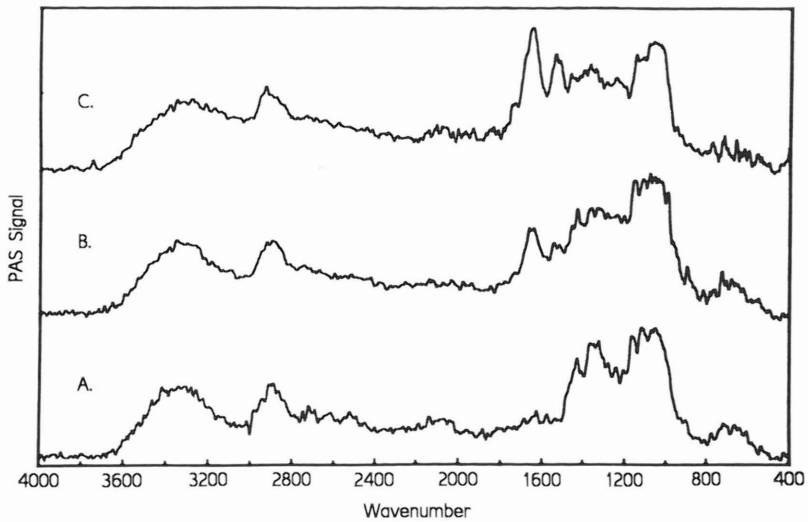

FIG. 1. FTIR–PAS signal versus dry weight of *P. chrysosporium*. Spectra of cellulose filter paper with deposits of (A) zero, (B) 0.67 mg, and (C) 3.20 mg fungal weight. PAS signals are in arbitrary units.

RESULTS AND DISCUSSION

As a point of departure from the previous work (14), consider the reported FTIR–PAS measurement of simulated growth of the white-rot fungus *Phanerochaete chrysosporium* on cellulose, shown in Fig. 1. Samples were prepared by depositing known dry weights of the fungus onto cellulose filter paper as in the present work but without an internal standard. The lower trace (A) in Fig. 1 is an FTIR–PAS spectrum of the cellulose filter showing the characteristic hydroxyl absorption band centered at 3338 cm^{-1}. The middle and upper traces (B and C) are FTIR–PAS spectra of cellulose filters onto which *P. chrysosporium* was deposited in 0.67- and 3.20-mg fungal dry weights, respectively. The three spectra show the appearance and increase of infrared bands from amide I (1656 cm^{-1}) and amide II (1538 cm^{-1}) absorptions from the protein in the fungus. The curve in Fig. 2 was generated from a series of such FTIR–PAS spectra taken over a wide range of fungal weights from samples of *P. chrysosporium* populations actually growing on cellulose. As is customary where no internal standard is present in the samples, all spectra were routinely normalized (15, 16) to the hydroxyl peak of the cellulose for comparison of the amide peaks. Judging from the scatter in the data (Fig. 2), the measurement error by this method was as high as 40%, particularly at the lower levels of fungal mass where the PAS response is still putatively quantitative. The saturation plateau in the PAS signal seen at the higher fungal weights has been attributed to mycelial layering (14,17), which limits penetration of incident radiation and thus sets an upper limit to the thickness of fungal mass that can be assayed by FTIR–PAS.

The *P. chrysosporium* results exemplify some of the most difficult problems of those that still beset quantitative photoacoustic spectroscopy, namely, the lack of reproducibility caused by instrumental artifacts, signal amplitude distortion introduced by the inherent variation in modulation frequency across the spectral range (18), and differences in morphology and particle size between samples (19). In efforts

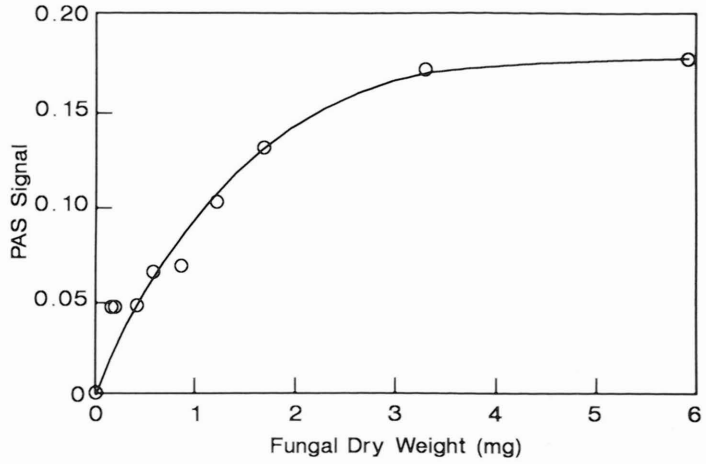

FIG. 2. PAS signal (arbitrary units) from amide I absorption (1656 cm^{-1}) versus fungal dry weight. All spectra were normalized to the cellulose OH peak (3338 cm^{-1}).

to improve the reliability of quantitative data, the present experiments were undertaken to determine the feasibility of employing an internal standard for FTIR–PAS as is commonly used in transmission spectrometry.

An internal standard suitable for solid-state PAS of microorganisms must be a stable compound that gives an adequate and distinctive photoacoustic signal in a solid matrix but does not interfere with the amide absorptions of the protein being measured. It was found that PAN, a low-molecular-weight polyacrylonitrile, provides an ideal internal standard by the simple physical treatment described in the above methods. The chemical structure of PAN is shown in Fig. 4A. The nitrile groups in PAN produce a strong FTIR–PAS signal which appears as a sharp peak (2243 cm^{-1}) isolated far from any significant absorption bands from protein, lipid, or carbohydrate present in biomaterials. Indeed, the nitrile absorption, which arises from stretching of the carbon–nitrogen triple bond, is fortuitously remote from the region of most infrared absorptions found in nature. Hence the nitrile band in PAN does not interfere with other absorption bands of biological interest, and provides a stable reference peak on which measured FTIR–PAS spectra can be normalized so that accurate and reliable quantitative data can be extracted.

To use PAN as the internal standard for solid samples it is necessary to pulverize the powder either directly into the sample or indirectly in water to be added to the sample prior to analysis. In the present work PAN was deposited from suspension onto Durapore filters together with the protein or microorganism under study. Figure 3A shows a scanning electron microscope (SEM) enlargement (300×) of the pulverized PAN particles on the surface of a Durapore filter. The particles range from irregular flakes to fluffy cotton-like bodies which average about 5 μm in diameter. Particle uniformity would presumably be dependent upon the pulverization time or conditions and may be adjustable, but this was not attempted in these early studies. Figure 3B shows a SEM enlargement (1000×) of a 60:40 dry weight mixture of *S. cerevisiae* and PAN on Durapore filter paper. The average PAN particles are clearly larger than the yeast cells. Although this would seem inappropriate for an internal standard (19),

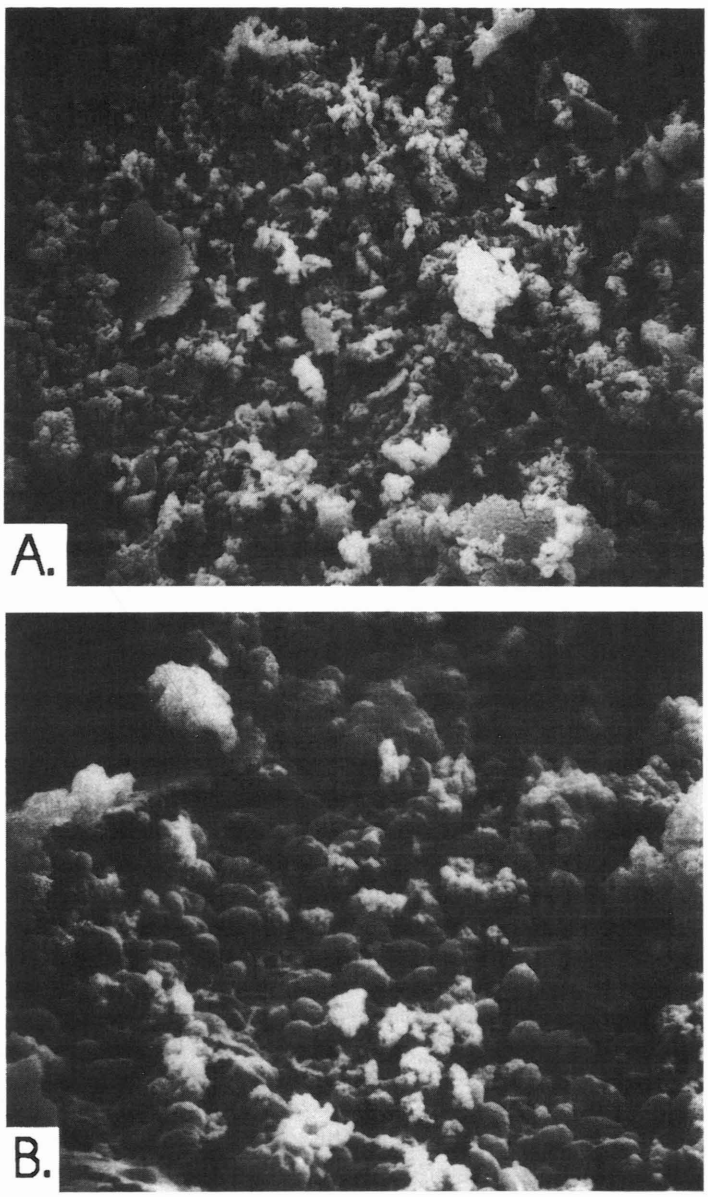

FIG. 3. (A) SEM enlargement (300X) of pulverized PAN particles on the surface of Durapore (polyvinylidene difluoride) filter paper. (B) SEM enlargement (1000X) of a 60:40 dry weight mixture of *S. cerevisiae* and PAN on Durapore filter.

it did not prove to be disadvantageous, as the following FTIR–PAS results demonstrate. Nevertheless, further study of the effects of the PAN morphology and particle size is needed and is currently in progress.

Three FTIR–PAS spectra are plotted in Fig. 4. The spectrum in Fig. 4A is of PAN itself. The nitrile absorption stands out as the solitary peak at 2243 cm^{-1}. Figure 4B shows the spectrum of PAN on a Durapore filter wherein absorption peaks from the

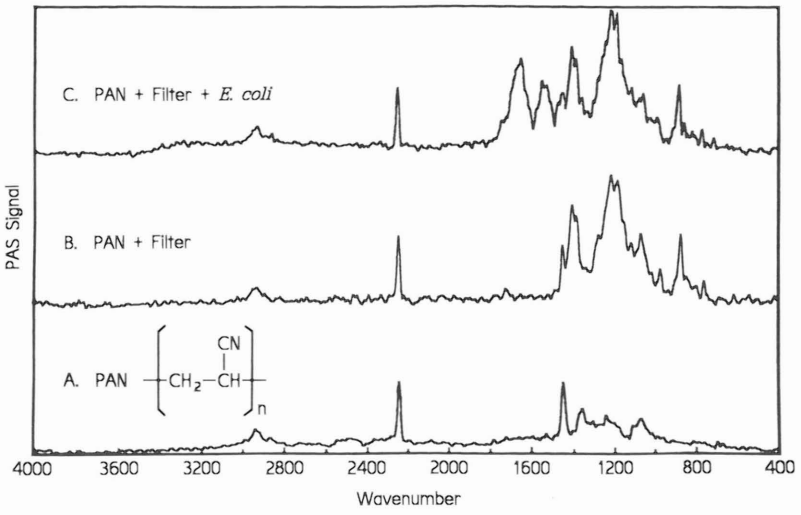

FIG. 4. (A) FTIR–PAS spectrum of polyacrylonitrile (PAN) showing its chemical structure in the annotation. (B) Spectrum of PAN on Durapore (polyvinylidene difluoride) filter. (C) FTIR–PAS spectrum of both *E. coli* and PAN on Durapore filter.

polyvinylidene difluoride filter material also appear. The spectrum in Fig. 4C is of both *E. coli* and PAN on a Durapore filter. Amide I and amide II peaks from the bacterial protein are clearly evident at 1656 and 1538 cm^{-1}, well separated from the nitrile peak. These spectra dramatically show that the nitrile absorption in PAN provides a distinct reference peak for normalizing the growth of protein amide absorptions. A material that gives such an ideal band for normalization of spectra and can be so readily incorporated into solid samples at any desired concentration provides a unique advantage for PAS analyses. Its use obviates arbitrary selections of reference bands that may prove to be unstable due to the contributions of overlapping analyte bands, as with the cellulose hydroxyl peak (Fig. 1) in the previous work.

In order to test the reproducibility of FTIR–PAS using PAN for quantitative assays of microbial mass on solid surfaces, four proteins and four microorganisms were selected for analysis to cover a broad range of common species and morphologies. Typical results of the assays are presented in Fig. 5. These are plots of FTIR–PAS signal versus protein content of samples containing a known weight (41.6 μg) of PAN. The protein levels ranged from zero in the reference sample to 170 μg in the test samples. The data points represent the PAS signal intensities of the amide I band for all protein levels after normalization to the nitrile peak of PAN in the reference sample.

In a comparison of Fig. 5 with Fig. 2, the similarity in the overall profiles is immediately seen, as all curves exhibit the expected saturation plateau at high protein levels. However, it is apparent from Figs. 5A and 5B that the plateau is reached at lower protein levels with the microorganisms than with the proteins themselves. This illustrates the fact that dry microorganisms contain about 70% nonprotein biomass, which adds to microbial layering and thus accelerates the saturation effect. Furthermore, when Fig. 5B is compared with Fig. 2, it is evident that the saturation effect occurred in both cases, but it appeared at considerably higher microbial weights when the microbes were naturally grown on the substrate than when they were artificially

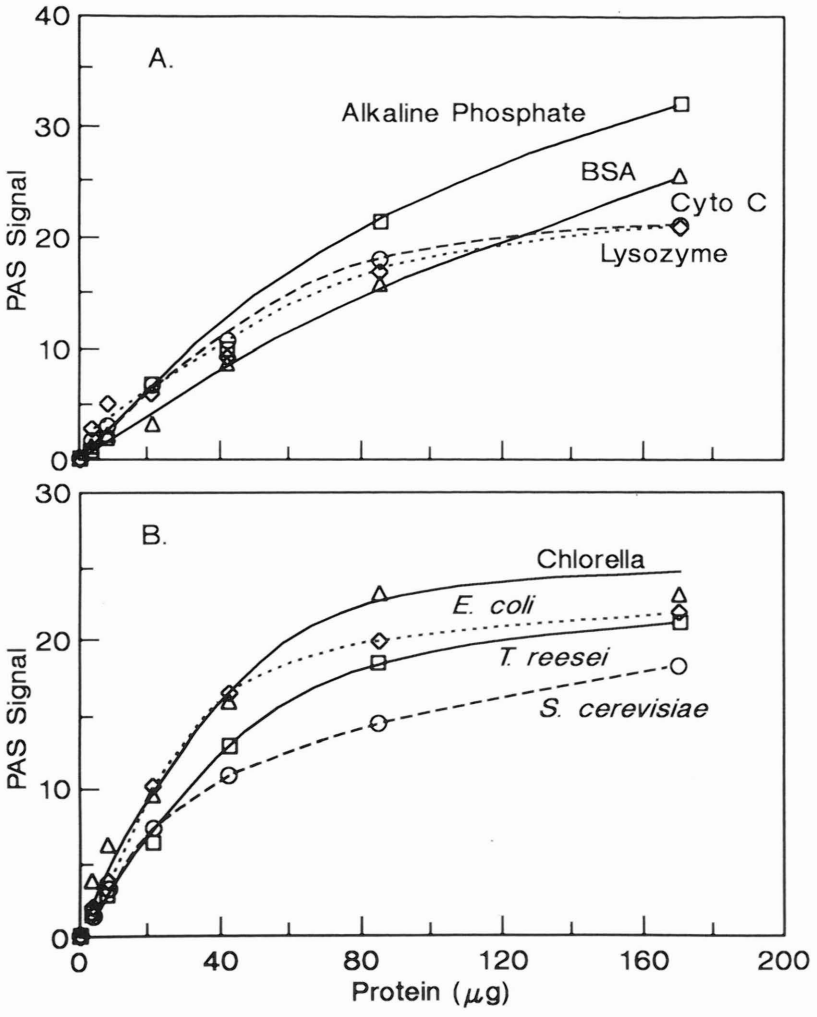

FIG. 5. FTIR–PAS signal (arbitrary units) from amide I (1656 cm^{-1}) versus protein content of samples containing 41.6 μg polyacrylonitrile as an internal standard. (A) Assays of four different proteins. (B) Assays of four species of microorganisms.

deposited onto the surface. In this case *P. chrysosporium* grows not only on the surface of the filter paper but, in using the cellulose as an energy source, the fungus also proliferates well into the fibrous matrix, thereby delaying the onset of saturation from mycelial layering. Hence, the upper limit of detection by FTIR–PAS should be substantially higher in natural population growth than in these simulation experiments, which were designed primarily to test the reproducibility of the method using the PAN internal standard. The lower limit of detection by FTIR–PAS in this work was comparable to that previously reported (14), about 2 parts microbes per thousand of substrate.

There is virtually no comparison, however, between the present data and the previous data when the reproducibility of the FTIR–PAS measurements is considered. As

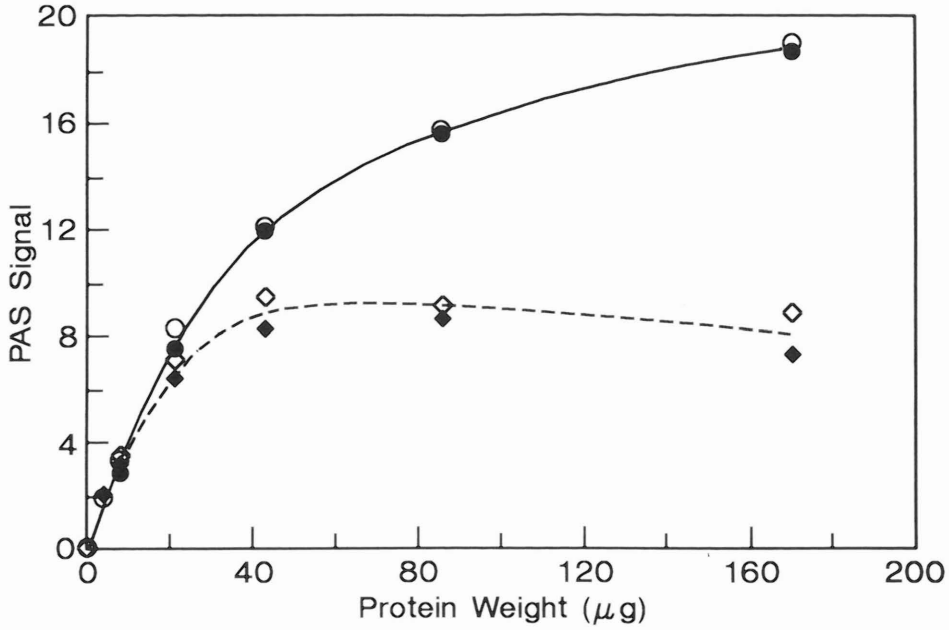

FIG. 6. FTIR–PAS signal (arbitrary units) from amide I (1660 cm^{-1}) versus protein content of samples (—) with normalization to polyacrylonitrile and (---) without normalization for two series tested in separate experiments over 2 days: (○) Series-1 normalized to PAN; (◇) Series-1 without normalization; (●) Series-2 normalized to PAN; (◆) Series-2 without normalization.

can be seen in Fig. 5B, the PAS signals from the amide I band vary smoothly with the protein contents of six different populations of each microorganism. This improved correlation can only be attributed to the use of the PAN internal standard, which enabled PAS measurements with less than 10% error. While it is not yet the epitome of analytical precision, this is a remarkable degree of accuracy for the fledgling photoacoustic spectroscopy.

Even stronger evidence of the value of PAN as an internal standard is shown in Fig. 6. Two series of data are plotted from PAS spectra of *S. cerevisiae* samples prepared and tested in separate experiments over 2 consecutive days. The first and second series were identical except for experimental error and instrumental variations over the 2 days. Since both sample sets contained the same concentration of PAN, it was possible to compare the variation between PAS spectra of the two series with and without normalization to the internal standard. In Fig. 6 the PAS signals from the series-1 and series-2 spectra without normalization show considerable differences, especially at the higher protein levels. PAS signals from these unnormalized spectra reached a saturation plateau at 50 μg protein. The plots are fairly smooth, showing that the variation within each series was not large but that the variation between series was significant (relative standard error = 0.0692). This indicates that human errors and instrumental fluctuations were larger between experiments than within each experiment, as would be expected.

However, as is also shown in Fig. 6, data from the same two series of PAS spectra plotted after normalization to the PAN internal standard revealed no significant differences between the plots for the series-1 and series-2 experiments. Variation in

the data was indeed reduced (relative standard error = 0.0462). But, more importantly, the normalized curve did not reach a saturation plateau within the protein range measured, as did the unnormalized curve. This suggests that the PAN internal standard not only reduces the effects of experimental and instrumental variations but also greatly extends the range of measurement of protein mass by PAS.

The results presented here clearly demonstrate the potential utility of PAN as an internal standard for quantitative FTIR–PAS analyses of biomass, and constitute a significant advance toward the goal (14) of practical application of the new technique to solid-state assays of microorganisms used in the bioproduction of drugs, hormones, and other biological agents.

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